Investigation of Novel Curcumin Analogs as Antimalarials

A Senior Research Thesis

Presented in partial fulfillment of the requirements for graduation with *research distinction* in Biomedical Science in the School of Health and Rehabilitation Sciences at The Ohio State University

By

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ABSTRACT

There are over two hundred million cases of malaria each year. Although there are effective treatments, drug resistant strains are emerging that make discovery of new antimalarials an important goal. We have developed a semi-automated assay platform to screen novel compounds for their ability to kill *Plasmodium falciparum*, the parasite that causes the most deadly form of human malaria. This assay was used to screen novel analogs of curcumin, a component of the spice turmeric, which is known to have antimalarial activity. Several of these analogs were found to inhibit parasite growth at low-micromolar to sub-micromolar concentrations. The compounds in this collection represent eight structural classes, and a structure-activity relationship analysis indicates a class-specific potency. Monocarbonyls had relatively high potency, while coumarins had very little activity against the parasite. The heteroaromatic and aromatic class of compounds are the most promising for future study because compounds in this class had very little activity against mammalian cell lines, indicating high selectivity for the parasite. Curcumin inhibits parasite growth at an IC_{50} of 6.06 μM while the most promising and selective compound in this screen, compound 47, is an aromatic compound with an IC_{50} of 0.512μM. We have developed an assay to investigate interactions between these compounds and current antimalarials in combination, and we hope to determine whether the promising compounds may be used effectively in combination with other existing antimalarials.

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CHAPTER 1

INTRODUCTION

Malaria is a devastating disease in many developing countries that infects over two hundred million people per year, killing an estimated 655,000 people in 2010 (1). It is the second leading cause of death from infectious disease in Africa, where 89% of worldwide malaria deaths occur (Figure 1) (2).

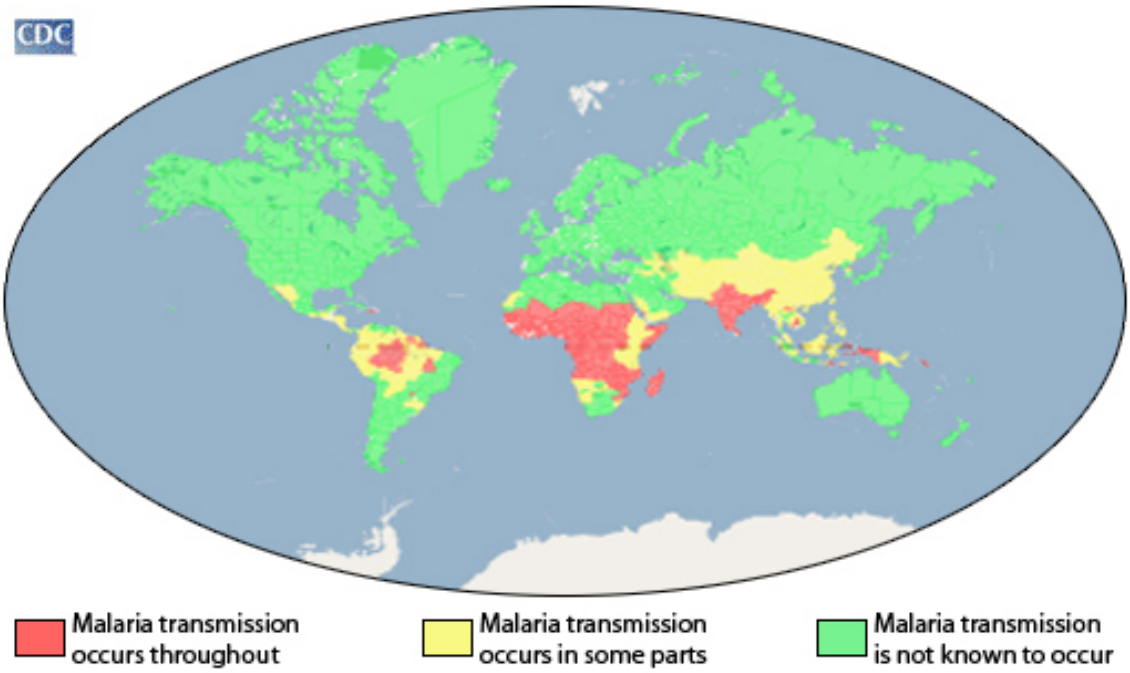


Figure 1. Geographical distribution of malaria transmission (2).

Plasmodium falciparum is the protozoan parasite that causes the most deadly form of human malaria. It is transmitted by the female *Anopheles* mosquito. Parasites are passed into the human blood stream by the mosquito, where they first replicate in hepatocytes. The parasite then goes through asexual replication cycles in the erythrocytes. The parasites mature from ring state to trophozoites to schizonts, and mature schizonts rupture the erythrocyte, releasing merozoites that infect new erythrocytes. Parasites can also differentiate into gametocytes, which

are ingested by the Anopheles mosquito during a blood meal. The blood stages of the parasite life cycle are responsible for the symptomatic stages of malaria (Figure 2).

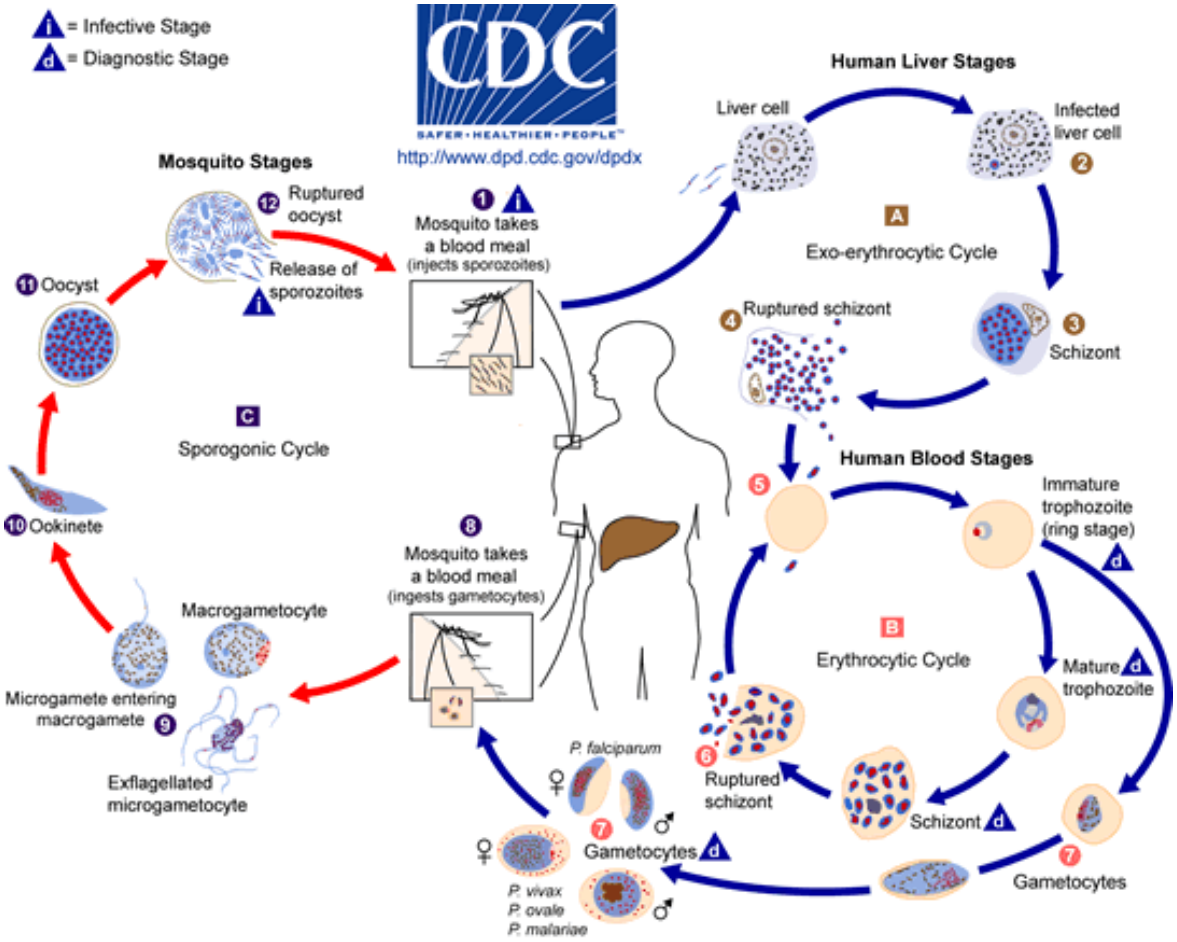


Figure 2. Life cycle of *Plasmodium falciparum* (2). The sporogonic cycle occurs in the *Anopheles* mosquito, the exo-erythrocytic cycle occurs in the human liver, and the erythrocytic cycle is the diagnostic stage, which is responsible for the symptoms of malaria.

The symptoms of uncomplicated malaria include fever, chills, headache, sweats, body aches, nausea, and malaise. The classical malaria attack consists of three stages: a cold stage with chills and shivering, a hot stage with fever, vomiting, and sometimes seizures, and a sweating stage characterized by sweating, returning to normal temperature, and tiredness. The whole attack typically lasts 6-10 hours. Severe malaria is much more serious and can include

cerebral malaria, anemia, acute respiratory distress syndrome, low blood pressure, and kidney failure (2).

Although effective drugs are available for malaria, the emergence of drug resistance makes treatment a challenge and necessitates the development of new antimalarials. Resistance, increased tolerance, or treatment failure has been documented for every antimalarial on the market, including recent reports of resistance to artemisinin in Cambodia and Thailand (3). Artemisinin combination therapy is currently the first-line treatment recommended by the World Health Organization (WHO). Given this developing resistance to even the most effective and widely used antimalarials, drug discovery has an important role in combating malaria.

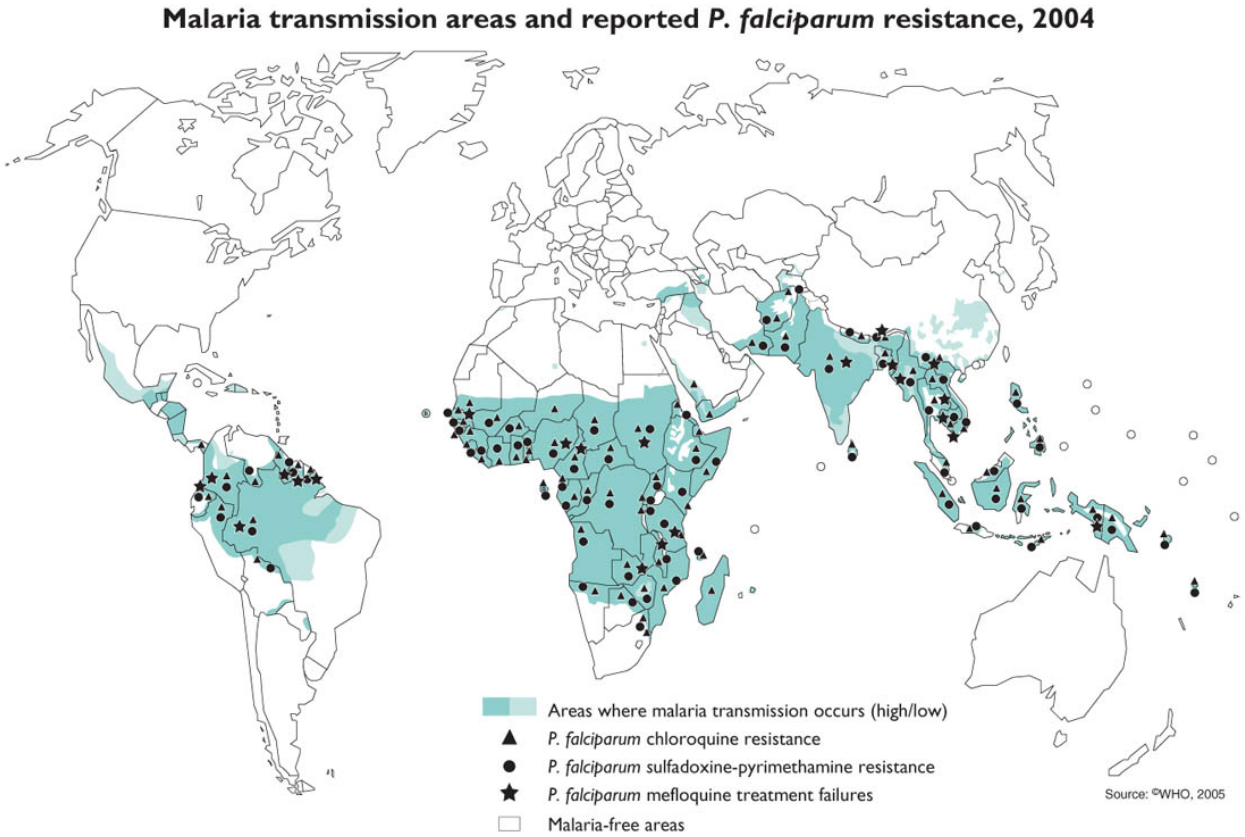


Figure 3. *Plasmodium falciparum* drug resistance (4). Chloroquine, sulfadoxine-pyrimethamine, and mefloquine resistance is widespread in malaria-endemic areas.

Curcumin is the component of the spice turmeric that gives it its yellow color. It is obtained from the root of the plant *Curcuma longa*, and has been used in traditional medicine for

many purposes including liver disease, arthritis, and insect bites. It has been shown to have activity against both chloroquine (CQ) sensitive and chloroquine resistant parasites (5). In addition to its antimalarial properties, curcumin has been shown to suppress growth of pancreatic and breast cancer cells (6). The Fuchs lab in The Ohio State University College of Pharmacy has created a library of curcumin analogs. Given the known antimalarial properties of curcumin, we screened this library in order to determine of any of the analogs are more potent than curcumin itself.

CHAPTER 2

MATERIALS AND METHODS

Parasite culture

Asynchronous 3D7 MR4 parasites were co-cultured with human O-positive erythrocytes (Interstate Blood Bank) in 12 mL volumes at 2% hematocrit. The media was RPMI 1640 with 0.5% Albumax (Gibco), 11.1mM glucose, 26.8mM $NaHCO_3$, 50µg/mL hypoxanthene, and 10µg/mL gentamicin (Sigma). The cultures were incubated at 37ºC in 5% O_2, 5% CO_2, and 90% N_2.

IC_{50} Assay

The IC_{50} assays were performed in a 384-well plate using optimized conditions for parasite growth in small volumes (75µL/well, 1% hematocrit, 1-1.5% initial parasitemia). 37.5µL of RPMI was added to the plate, and the drugs were serially diluted using 2.5-fold dilution with a Biomek 2000 to give a concentration range of 80µM to 0.131µM. 37.5µL of asynchronous culture (2% hematocrit, 1-1.5% parasitemia) was added to each well to give a final hematocrit of 1%, drug concentrations of 40µM to 0.065µM, 1-1.5% parasitemia, and maximum 0.4% DMSO. The cultures were incubated at 37ºC in 5% O_2, 5% CO_2, and 90% N_2. After 72

hours the cultures were resuspended and a 2.5µL sample was added to 50µL of 1.5µg/mL acridine orange in PBS and shaken for 30 seconds at 1500rpm. The parasitemia was determined using high-throughput flow cytometry with a BD FACS Canto II. IC_{50}s were calculated using the sigmoidal dose-response variable slope equation in GraphPad PRISM.

Isobologram

The isobologram assay was based on the fixed-ratio isobologram described in Fivelman et al (7) and was modified to be performed in 384-well plate format using the Biomek. IC_{50} assays were first dome to determine the IC_{50} of each drug. The two drugs were combined for the isobologram assay so that the IC_{50} fell at about the sixth twofold serial dilution. The combination solutions were prepared with fixed ratios (Figure 6A) so that the highest concentration of each drug was 0.50 µM (combination 1 for artemisinin, combination 10 for CQ). The combinations were placed in triplicate in columns 1 and 13 of a 384-well plate, with row P as a no drug control. Each combination was serially diluted into RPMI by the Biomek in a twofold serial dilution across a 1000-fold range, and culture was added using the conditions optimized for parasite growth in small volumes. The plate was incubated at 37ºC in 5% O_2, 5% CO_2, and 90% N_2. After 72 hours parasitemia was determined using flow cytometry as described above, and two fractional inhibitory concentrations were calculated for each combination using the concentration of a single drug. The fractional inhibitory concentration was calculated as IC_{50} of drug in combination divided by IC_{50} of drug alone. The isobologram was plotted with each drug's FIC_{50} on an axis, where points above the control line represent antagonism and points below the control line represent synergy.

CHAPTER 3

RESULTS

Assay Development

In order to screen compounds for their effectiveness at inhibiting *P. falciparum* growth, we developed a semi-automated assay. The assay is used to screen either ten or fifteen compounds at a time in a 384-well plate format. Optimal conditions for culture growth in small volumes were determined in order to provide the best environment for parasite growth for the assay. The four variables to be optimized for the assay are culture volume, initial parasitemia, incubation time, and hematocrit.

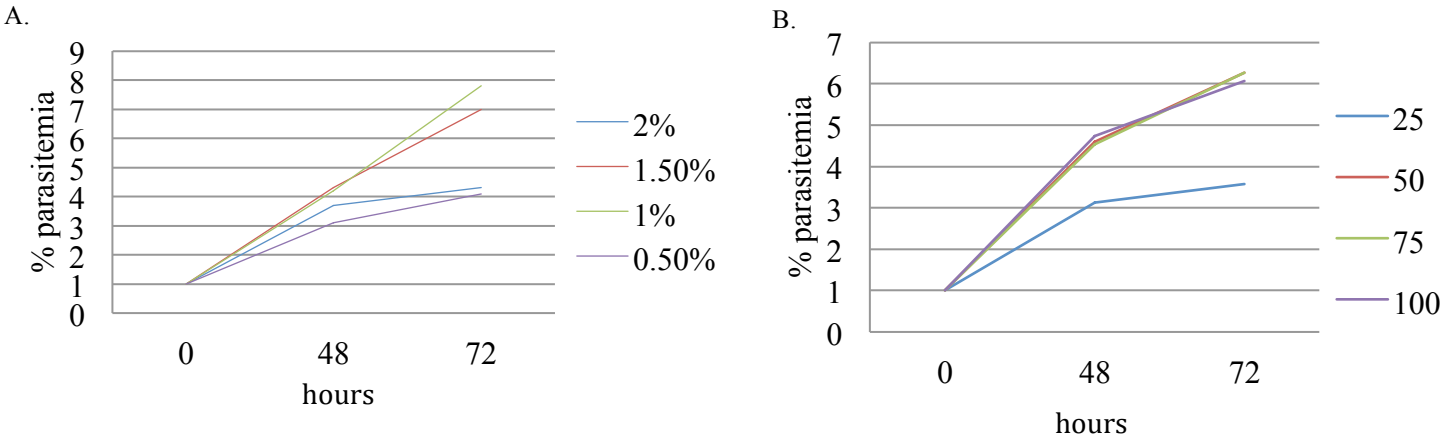


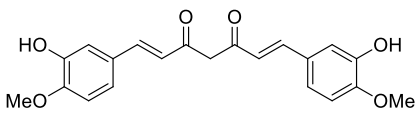
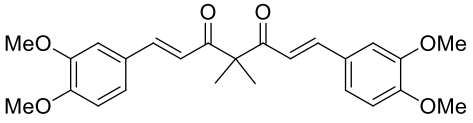
Figure 4. Optimization of *Plasmodium falciparum* growth assay conditions. (A) Culture growth with varied hematocrit. (B) Culture growth with varied volume (µL).

The optimal hematocrit (percent red blood cells by volume) for growth in small volumes was found to be 1%. Similar growth was observed in wells containing 50µL, 75µL, and 100µL of culture. 75µL per well was chosen for the assay due to concerns about evaporative loss over the incubation period with small volumes. The cycle time for 3D7 MR4 parasites is 34 hours, so an incubation length of 72 hours was chosen in order to make sure that all parasites went through

at least one full cycle. A 48-hour assay would also allow for at least one erythrocytic cycle, but 72 hours also allows for IC_{50} determination for drugs that display a delayed death phenotype. The initial parasitemia (percent parasitized red blood cells) was set at 1-1.5% for the assay in order to allow the parasites to grow to a point where a noticeable difference in parasitemia could be observed between treated and untreated cultures. Untreated cultures starting at 1-1.5% can grow to about 20% parasitemia over a 72-hour period. The compounds were dispensed over a range of concentrations using a Biomek 2000 liquid-handling robot and culture was added according to the optimized conditions. After incubation, parasitemia was determined by flow cytometry and dose-response curves were generated using GraphPad Prism software.

Screening and Structure-Activity Relationship Analysis

The Fuchs lab in The Ohio State University College of Pharmacy created a library of 109 novel curcumin analogs, which were screened against *P. falciparum* using the high-throughput approach described above to determine the IC_{50}, the concentration of drug that inhibits parasite growth by 50%. The semi-automated assay was used to generate dose-response curves and determine IC_{50}s for all 109 compounds. The Fuchs lab also divided the compounds into eight structural classes so that relationships between structure and activity could be more effectively studied (Table 1).

Class	Structure
Curcumin-like	**17** 
Dialkylated Curcumin	**54** 

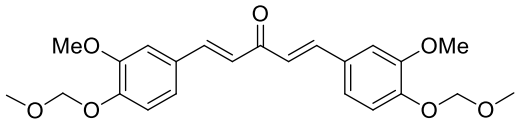
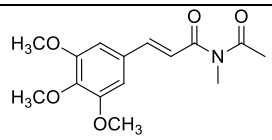
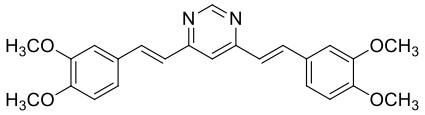
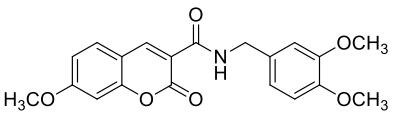
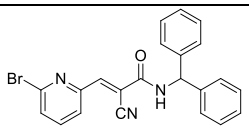
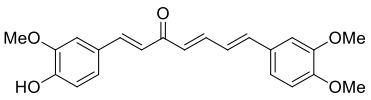
Monocarbonyls	13 
Imides	100 
Heteroaromatic/aromatic	30 
Coumarins	110 
Amides	84 
Miscellaneous	46 

Table 1. Representative structures for each class of curcumin analogs.

A structure-activity relationship (SAR) analysis shows that the dialkylated curcumin compounds and the monocarbonyls are very potent; many of the compounds in these categories had IC_{50}s lower than that of curcumin. The imides and coumarins show very little potency; almost all of these compounds had IC_{50}s that were greater than 40μM. The heteroaromatic/aromatic class was mostly not active, with the exception of compounds 30 and 47. There is some variation in the amides and curcumin-like compounds, but in general they are not very potent (Figure 5).

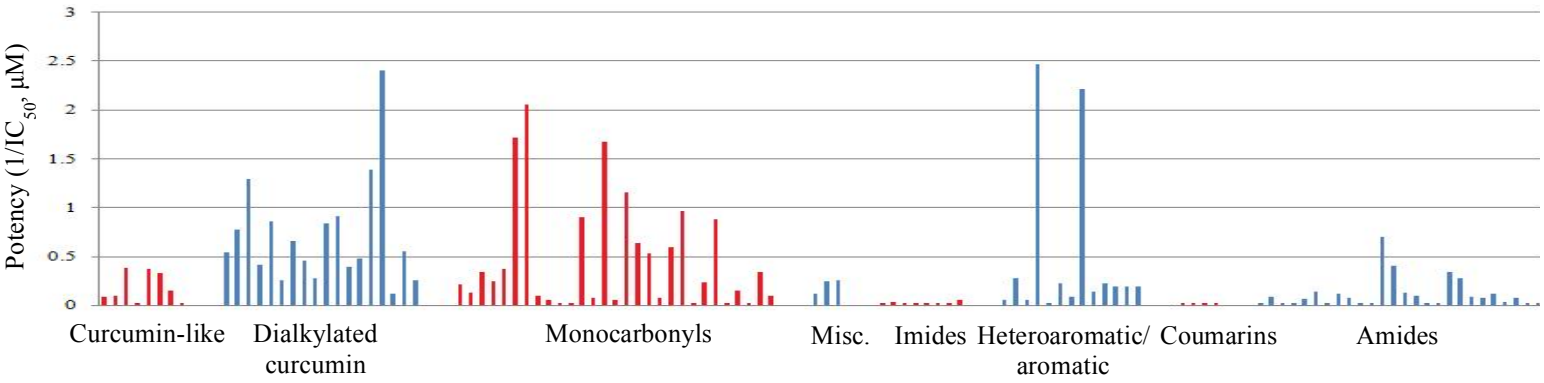
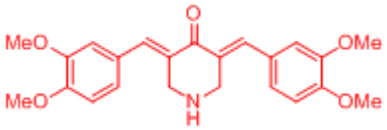
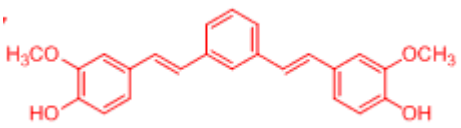


Figure 5. Differences in potency are dependent on the class of analogs.

Although all of the analogs are structurally based on curcumin, they have very different structures and widely varying IC_{50}s. The ten most potent compounds from the first screen were screened again to verify their potency. The two most potent, compounds 64 and 47, have similar IC_{50}s but come from different classes and have markedly different structures. Artemisinin, the current first-line treatment for malaria, has an IC_{50} of 0.010 μM. The lowest IC_{50} in this screen was compound 64, which has an IC_{50} of 0.488 μM (Table 2). Several compounds in the screen had IC_{50}s over 40 μM, while curcumin (compound 1) has an IC_{50} of 6.06 μM.

Compound	IC_{50} (μM)	Class	Structure
64	0.488 ± 0.32	Monocarbonyls	
47	0.512 ± 0.086	Heteroaromatic/aromatic	

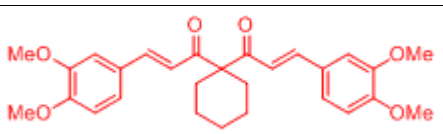
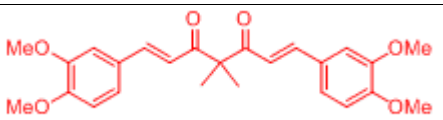
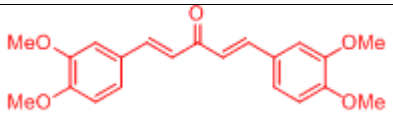
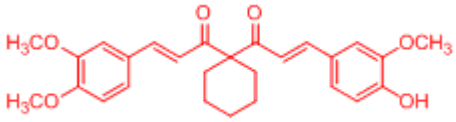
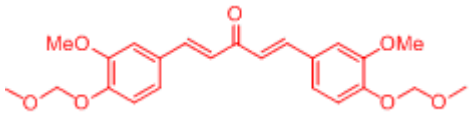
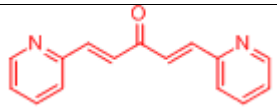
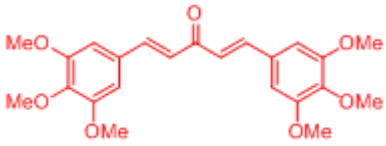
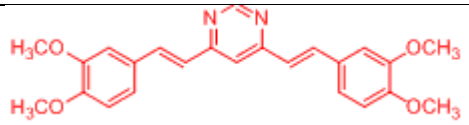
55	0.708 ± 0.41	Dialkylated curcumin	
54	1.011 ± 0.41	Dialkylated curcumin	
12	1.249 ± 0.88	Monocarbonyls	
36	1.287 ± 0.72	Dialkylated curcumin	
13	1.329 ± 1.2	Monocarbonyls	
32	1.352 ± 0.69	Monocarbonyls	
25	1.682 ± 1.5	Monocarbonyls	
30	1.824 ± 1.9	Heteroaromatic/aromatic	

Table 2. Top ten curcumin analogs and their IC_{50}s. The top ten compounds come from three different classes and have IC_{50}s below 2 μM. The IC_{50}s are the average of two different assays.

The top ten compounds come from three different categories, and have IC_{50}s in the low-micromolar to sub-micromolar range. The only categories with compounds in the top ten are

monocarbonyls, heteroaromatic/aromatic and dialkylated curcumin. In order to further prioritize compounds for their potential as an antimalarial the compounds' IC_{50}s against mammalian cell lines were compared. A good antimalarial should be selective for the parasite, meaning that it is toxic to the parasite and not to human cells. The comparison of IC_{50}s between mammalian cells and parasite cultures allows us to compare toxicity and identify compounds that are selective for the parasite. The heteroaromatic/aromatic class of compounds had low IC_{50}s against *P. falciparum* and high IC_{50}s against two mammalian cell lines, a prostate cancer cell line and a breast cancer cell line. The imide class of analogs, on the other hand, had very high IC_{50}s against *Plasmodium falciparum* and low IC_{50}s against the mammalian cell lines (Table 3).

Compound	**Class**	***Plasmodium* (IC_{50} µM)**	**DU-145 (IC_{50} µM)**	**MDA-MB-231 (IC_{50} µM)**
30	Heteroaromatic/aromatic	1.824 ± 1.9	16.1 ± 3.4	> 50
47	Heteroaromatic/aromatic	0.512 ± 0.086	>50	> 50
55	Dialkylated curcumin	0.708 ± 0.41	1.3 ± 0.2	1.9 ± 0.5
54	Dialkylated curcumin	1.011 ± 0.41	2.7 ± 1.2	-1.7 ± 0.5
36	Dialkylated curcumin	1.287 ± 0.72	4.7 ± 0.03	-6.5 ± 0.5
64	Monocarbonyls	0.488 ± 0.32	-0.5 ± 0.06	-1.6 ± 0.7
98	Imides	>40	2.3 ± 0.1	-1.7 ± 0.5
109	Imides	~16.60	4.1 ± 0.9	-1.6 ± 0.4

Table 3. A comparison of IC_{50}s for several compounds against *Plasmodium falciparum* and mammalian cell lines. The DU-145 (a prostate cancer cell line) and MDA-MB-231 (a breast cancer cell line) data came from the Fuchs lab in the OSU College of Pharmacy.

Since the parasite circulates in the bloodstream in infected individuals, it is also important to determine the toxicity of the drugs against erythrocytes. Four compounds were incubated with unparasitized erythrocytes at the IC_{90} concentration to determine if there was a significant change in hematocrit due to the effects of the drug (Table 4). The high concentration of the drug should allow for any toxicity to be apparent when the red blood cells are exposed to the drug for 72 hours. Although the hematocrit decreased over the course of 72 hours, the difference in

hematocrit is due to the natural breakdown of erythrocytes over time and not due to exposure to the drug.

Compound	IC_{90} (μM)	Initial hematocrit	72 hr. hematocrit
Curcumin	11.61	4.17%	3.51%
30	3.42		3.09%
64	1.13		3.86%
No drug	0		3.19%

Table 4. Hemolysis assay for selected compounds at the IC_{90}. Unparasitized red blood cells were incubated with a high concentration of drug, showing no difference in hemolysis between the treated and untreated erythrocytes.

Isobolograms

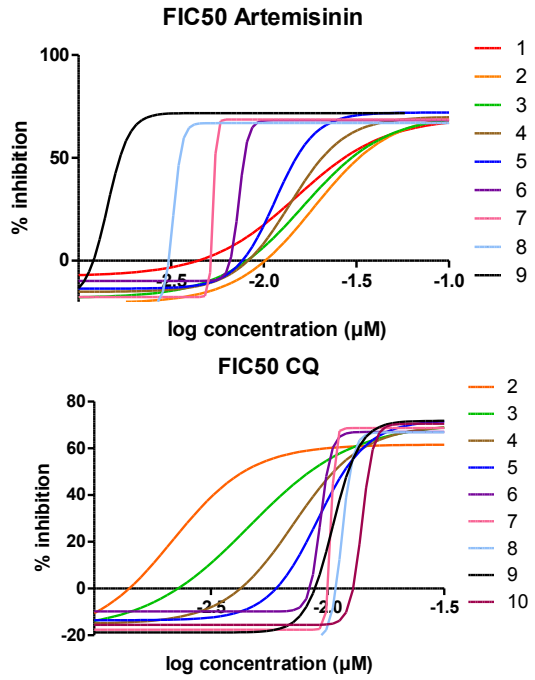
In order to study the interactions between two drugs, an assay called an isobologram is used. Additive effects have been reported between the current first-line antimalarial, artemisinin, and curcumin (8). The semi-automated assay for determining drug interactions was created based on the protocol described in Fivelman, et al (7). The isobologram is performed by combining two drugs in varying ratios (Figure 6A). Each combination is then serially diluted by the Biomek robot so that the IC_{50} values fall in the middle of the dilution. The combinations are incubated with parasite culture and inhibition is calculated in a manner similar to an IC_{50} assay for each drug individually, taking into account only the concentration of that drug in the combination (Figure 6B). The fractional IC_{50} (FIC_{50}) for each drug combination is calculated as the IC_{50} for that drug in the combination divided by the IC_{50} of the drug alone. The fractional IC_{50}s for each drug combination are plotted against each other, and the fractional IC_{50} represents the contribution to the total inhibition from the individual drug. A line is drawn on the graph

connecting a FIC_{50} of 1 for each drug. If the points are below 1 on the graph they represent synergy because there is more inhibition from the combination than can be accounted for by the two drugs individual effects. Points on the line indicate an additive effect, and points above the line indicate an antagonistic effect.

A.

Combination Solution	Ratio of Artemisinin to CQ	
	Artemisinin	CQ
1	9	0
2	8	1
3	7	2
4	6	3
5	5	4
6	4	5
7	3	6
8	2	7
9	1	8
10	0	9

B.



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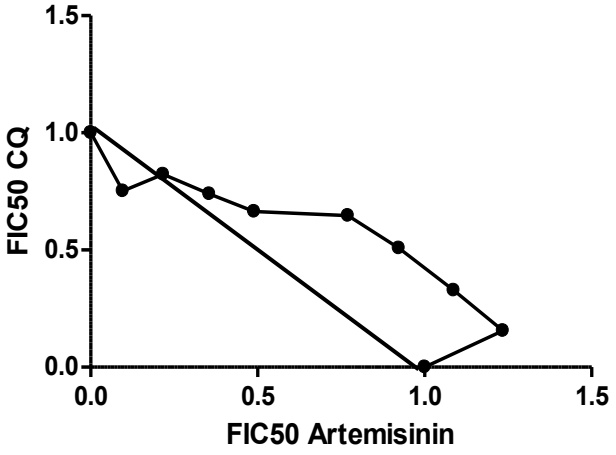

Figure 6. Fixed-ratio isobologram. (A) Ratio of drugs in each solution. (B) Fractional IC_{50}s for artemisinin and CQ. (C) Isobologram showing antagonism between artemisinin and CQ.

The points lie mainly above the line, indicating an antagonistic relationship between chloroquine (CQ) and artemisinin (Figure 6C). We have successfully used the semi-automated isobologram assay to demonstrate the expected antagonistic effects between artemisinin and chloroquine (9). This assay will soon be used to determine the interactions between promising curcumin analogs and artemisinin.

CHAPTER 4

DISCUSSION

We have carried out a multiple-step screening approach to determine which of the curcumin analogs in this library are promising drugs for future study. Initially, all 109 compounds were screened using a semi-automated assay where a Biomek 2000 liquid-handling robot dispenses compounds and culture. This allows for the assay to be carried out in a 384-well plate format. Dose-response curves were generated and IC_{50} values calculated for all of the compounds. Curcumin (compound 1) has an IC_{50} of 6.06 μM, and there were several compounds that had IC_{50}s lower than that of curcumin. The ten most potent compounds from the initial screen were screened again, and these ten compounds were considered the most promising for future study.

The Fuchs lab in the OSU College of Pharmacy divided the library into eight structural classes, with widely varying potencies. The monocarbonyls and dialkylated curcumin analogs were relatively potent, while the imides and coumarins had very little potency. The curcumin-like, amides, and miscellaneous compounds had intermediate potency. Interestingly, most of the compounds in the heteroaromatic/aromatic class had low to intermediate potency, with the exception of compounds 30 and 47. The compounds have similar structures, but compound 47 is an aromatic compound and compound 30 is a heteroaromatic compound. The structural feature

that makes these two compounds so much more potent than other heteroaromatic/aromatic compounds is unknown.

In order to determine which classes of compounds are most selective for the parasite, the IC_{50} against *Plasmodium falciparum* was compared to previously obtained data from the Fuchs lab on the IC_{50}s against two mammalian cell lines. Both potent aromatic/heteroaromatic compounds, 30 and 47, had very low IC_{50}s against *Plasmodium* and high IC_{50}s against the mammalian cells. The heteroaromatic/aromatic compounds selectively inhibit growth of the parasite, making them even more promising class of analogs. In contrast, the imides had high IC_{50}s against the parasite and low IC_{50}s against the mammalian cell lines. The monocarbonyls and dialkylated curcumin analogs had similar potency for all cell lines, indicating that they have a broad cytotoxic effect and are not specific. The effect of the drug on the erythrocyte was also considered. When nonparasitized red blood cells were incubated at the IC_{90} for 72 hours, there was no significant hemolytic effect from the drug as compared to a no-drug control.

In order to delay the emergence of drug resistance, combination therapies for malaria are preferred over monotherapies. The currently recommended combination therapy is artemisinin-based. Since additive effects have been reported between curcumin and artemisinin (8), determining the interactions between the promising compounds from this screen is important in further investigating the heteroaromatic/aromatic compounds. The isobologram assay for determining synergy, antagonism, or additive effects has been developed using the Biomek robot, and can soon be used for the curcumin analogs. The interactions between artemisinin and compounds 47 and 30 are of particular interest since they are the most promising of the curcumin analogs.

This screen has identified several compounds that inhibit parasite growth at low-micromolar or sub-micromolar concentrations, all of which have a lower IC_{50} than the parent compound, curcumin. One class of analogs in particular, heteroaromatic/aromatic compounds, included two drugs which were selective inhibitors of parasite growth with no adverse effects on mammalian cells. This promising class of compounds should be further investigated, especially to determine the mechanism of action and their effects on resistant parasites.

APPENDICES

Appendix A

Structures of all analogs

Curcumin-like: 8 compounds

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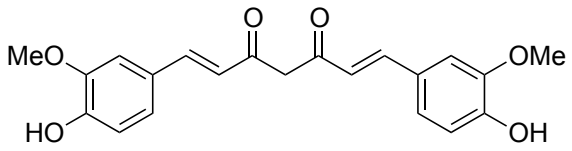

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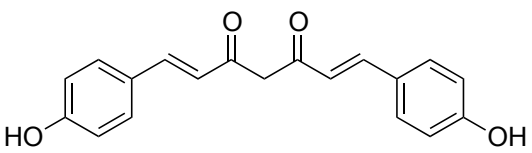

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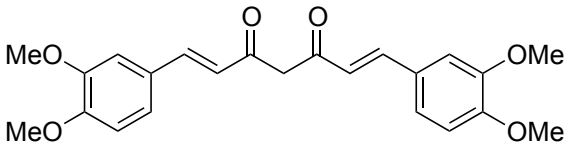

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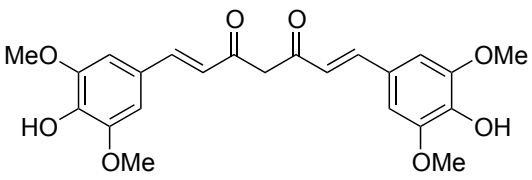

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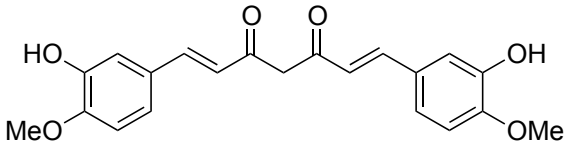

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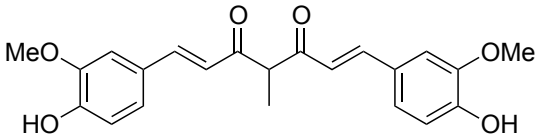

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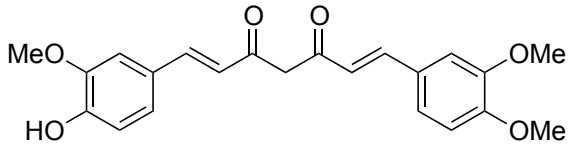

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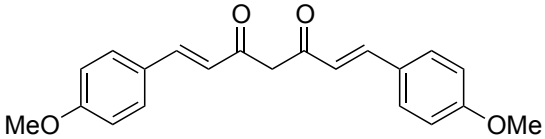

Dialkylated Curcumin: 18 compounds

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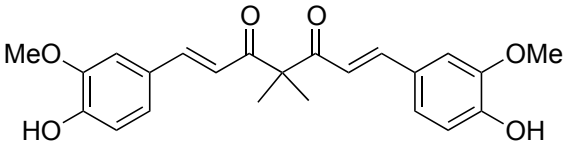

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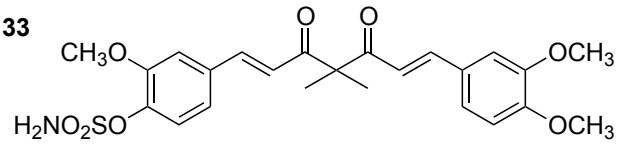

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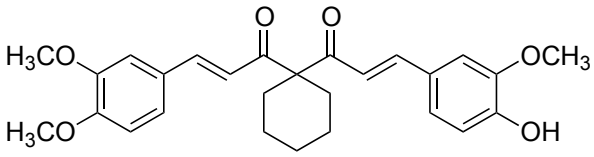

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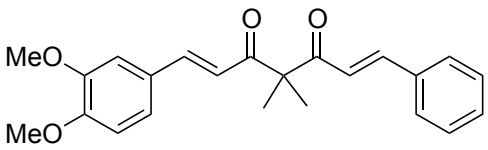

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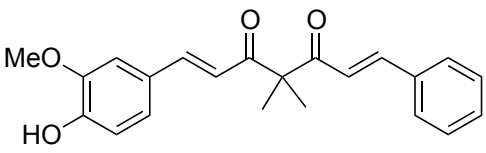

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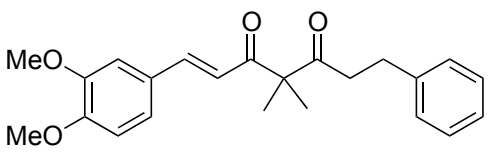

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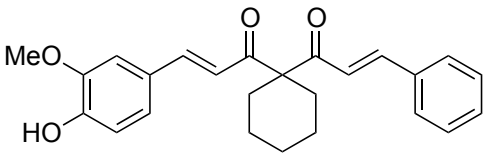

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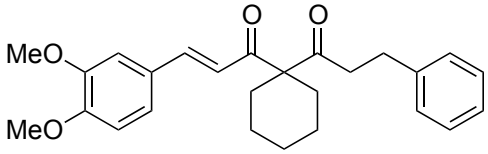

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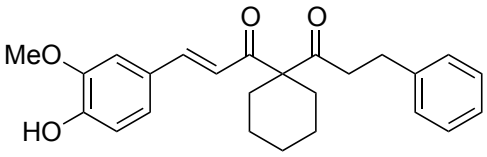

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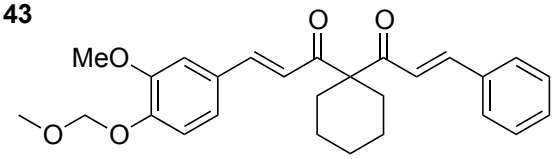

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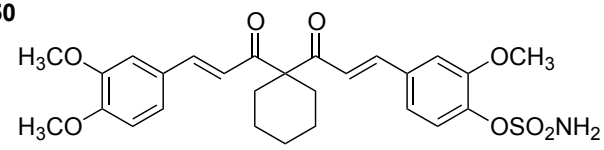

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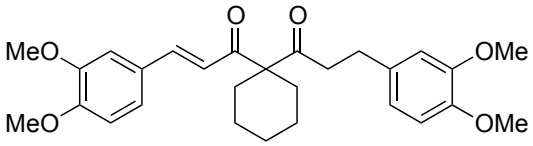

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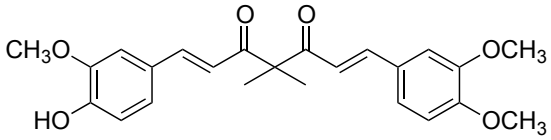

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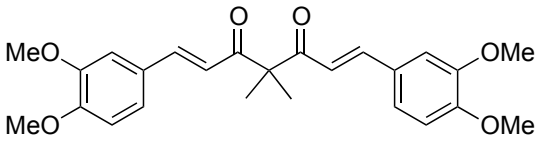

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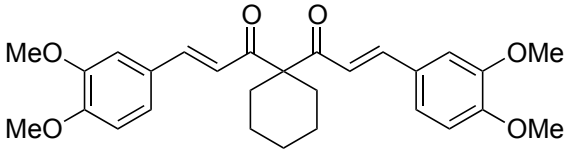

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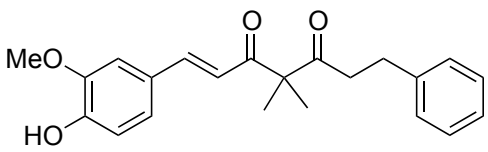

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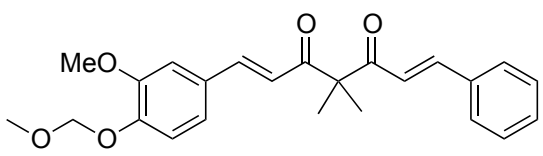

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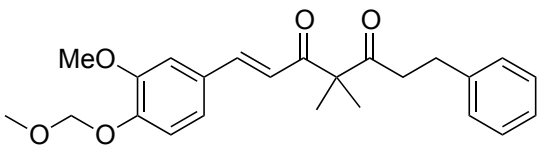

Heteroaromatic/aromatic: 13 compounds

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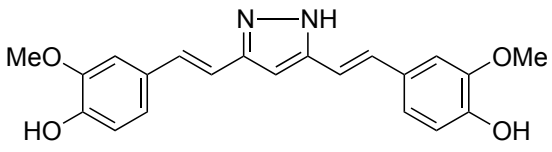

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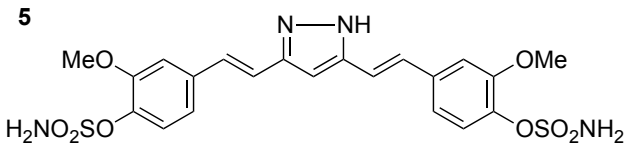

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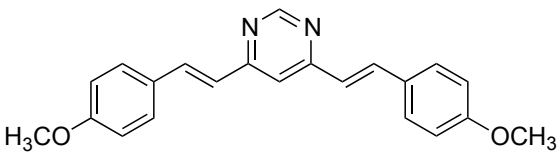

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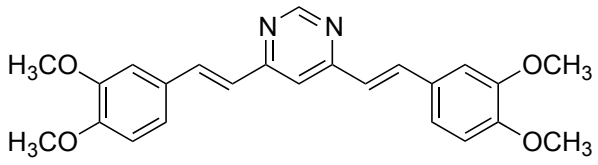

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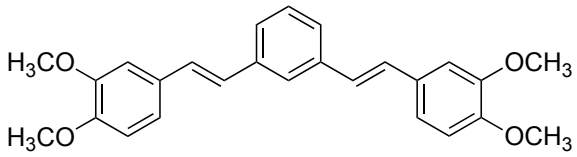

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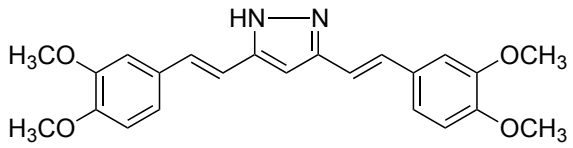

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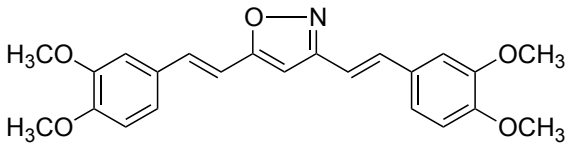

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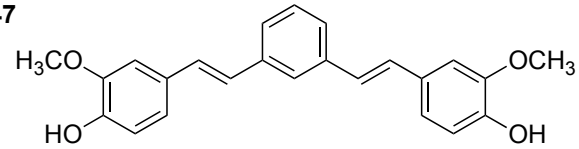

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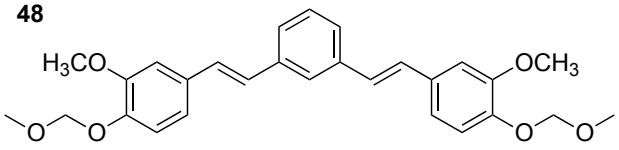

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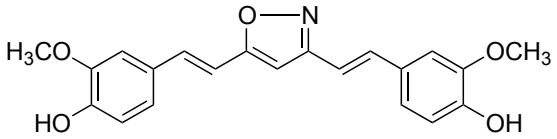

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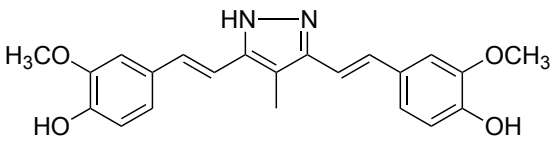

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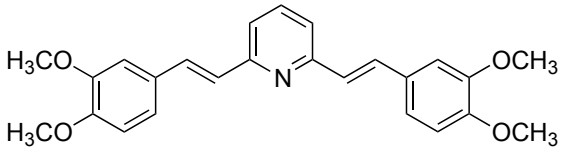

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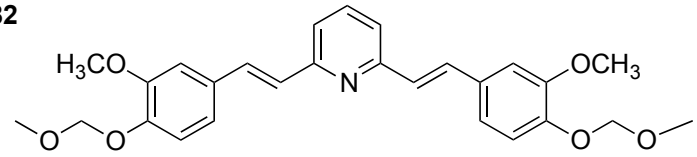

Monocarbonyls: 29 compounds

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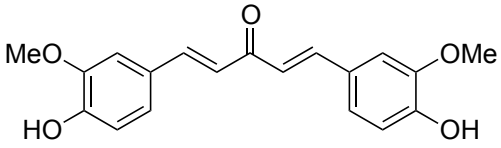

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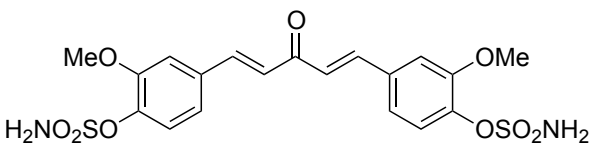

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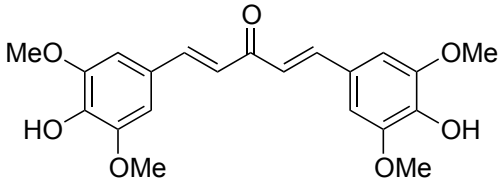

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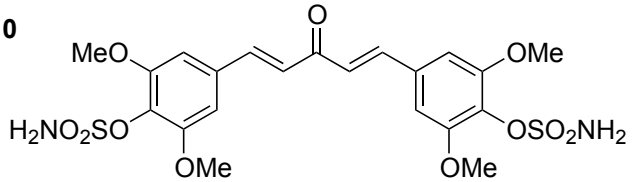

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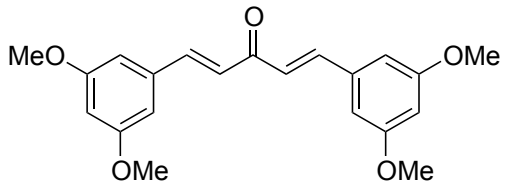

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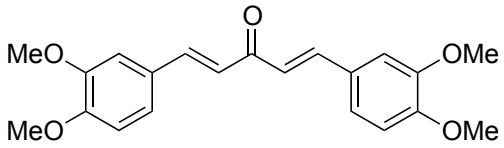

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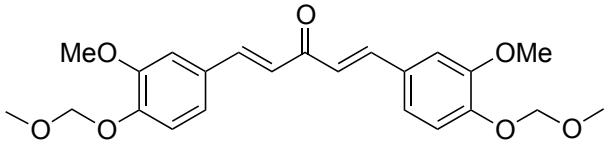

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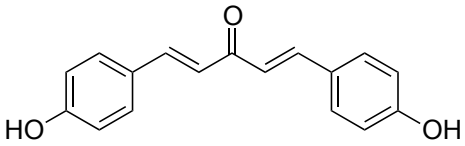

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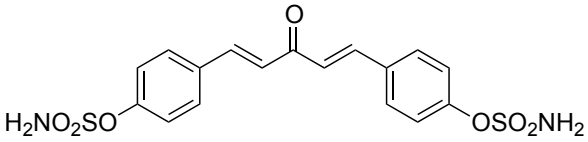

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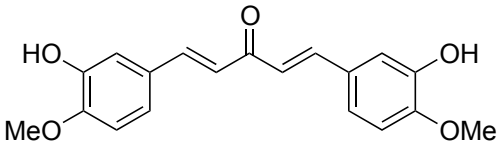

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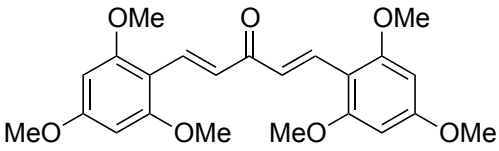

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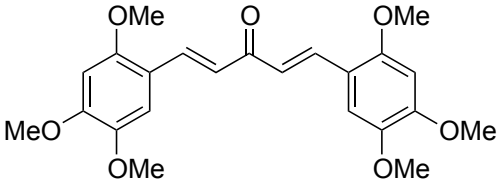

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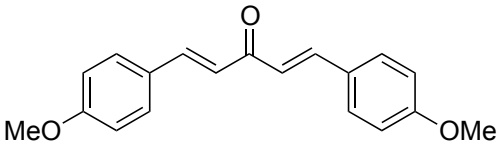

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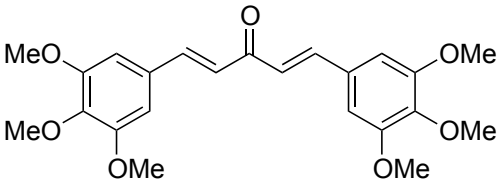

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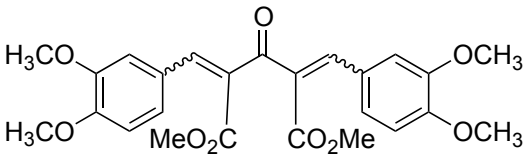

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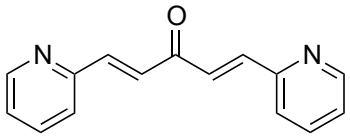

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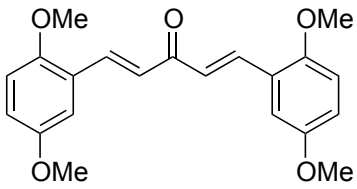

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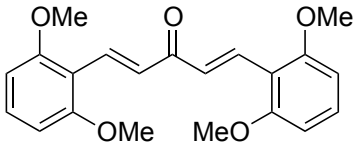

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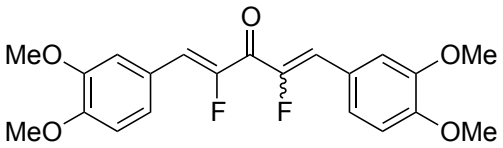

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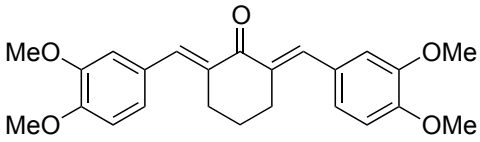

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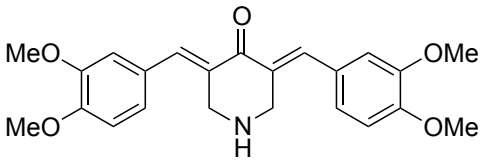

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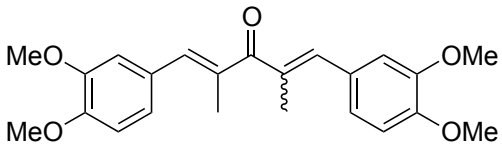

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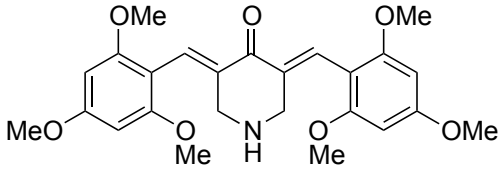

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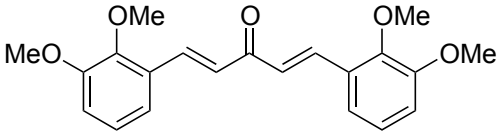

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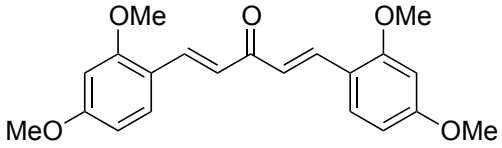

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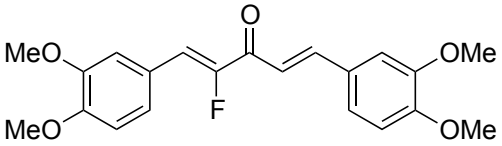

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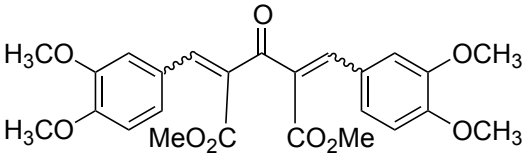

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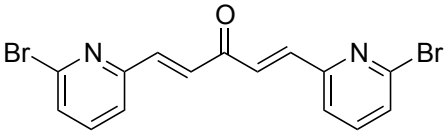

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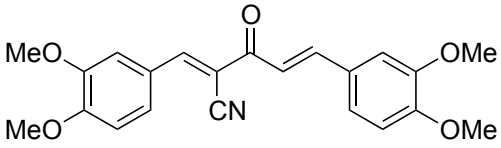

Amides: 26 compounds

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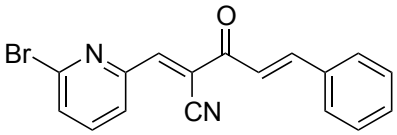

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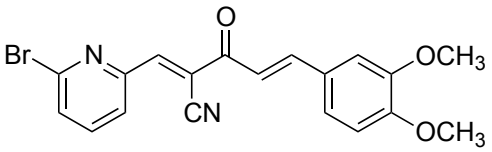

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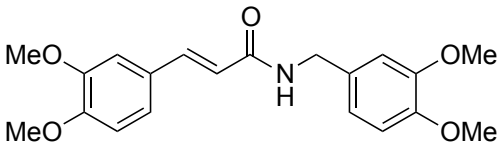

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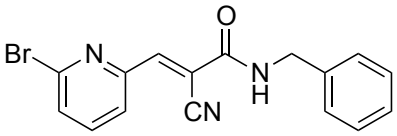

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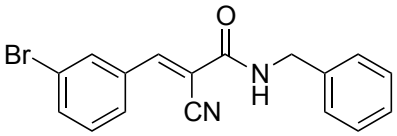

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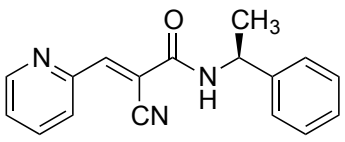

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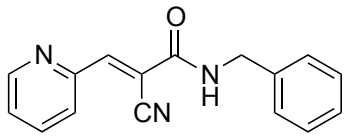

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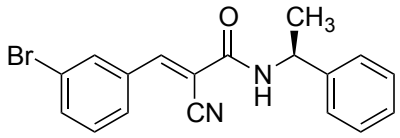

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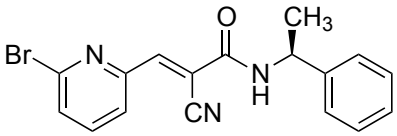

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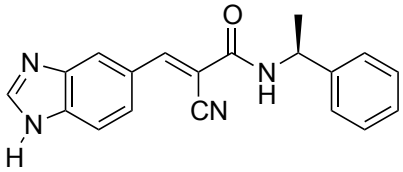

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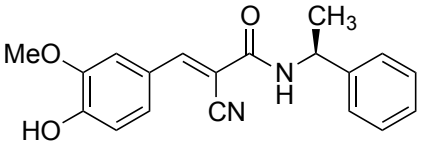

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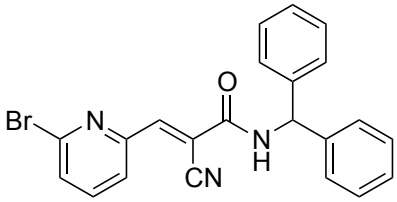

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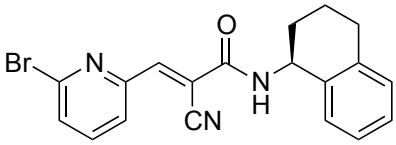

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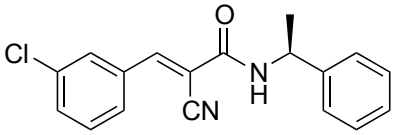

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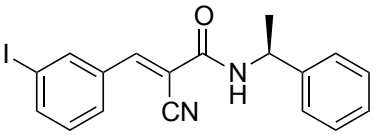

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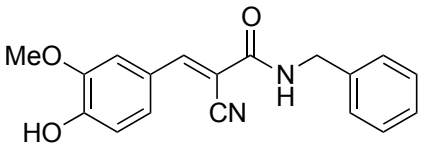

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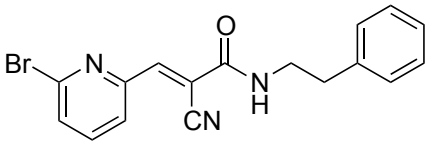

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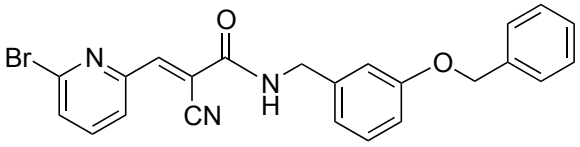

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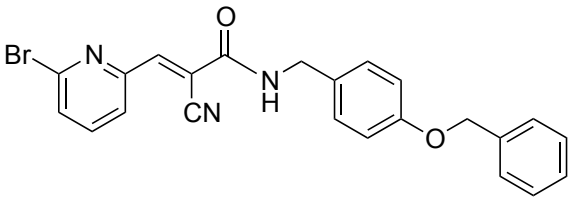

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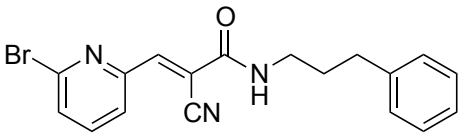

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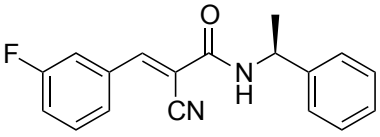

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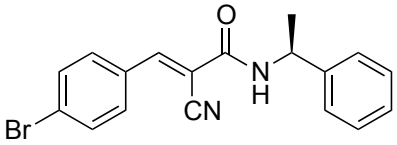

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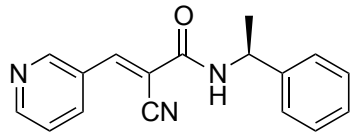

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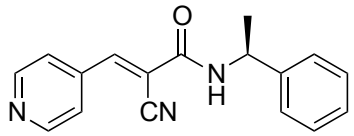

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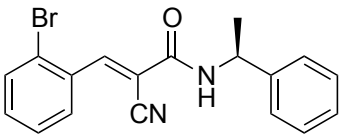

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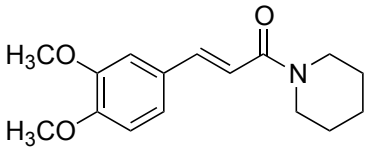
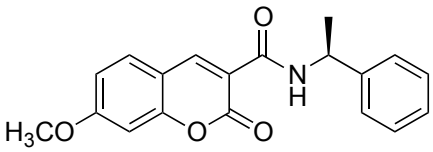


Coumarins: 4 compounds

104



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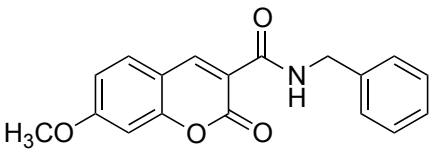

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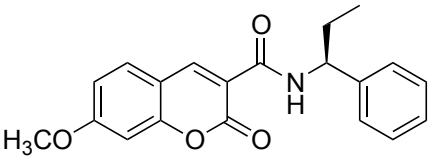

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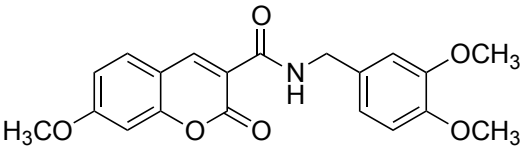
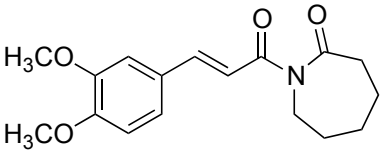


Imides: 8 compounds

98



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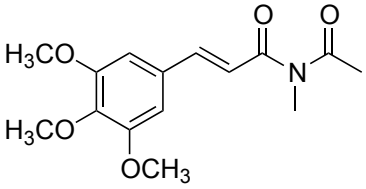

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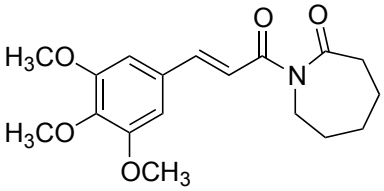

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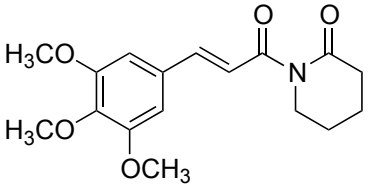

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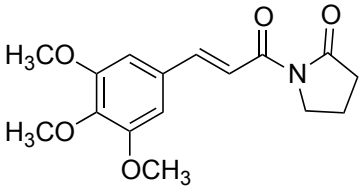

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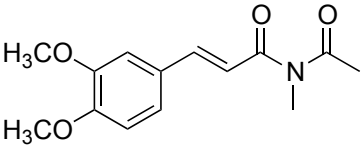

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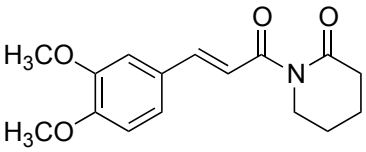

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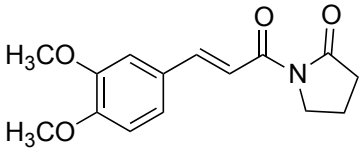
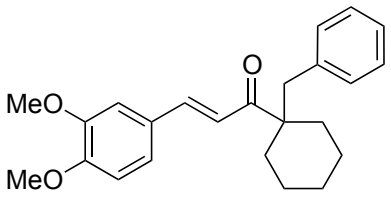


Miscellaneous: 3 compounds

44



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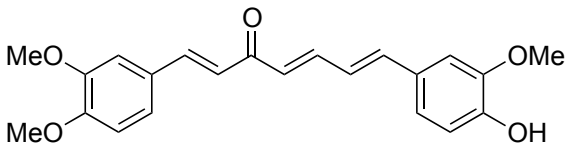

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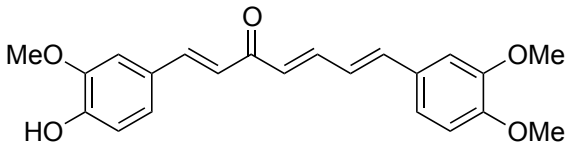

Note: compound 59 was a duplicate of one of the other compounds in the library and was removed.

Appendix B

IC_{50}s of all analogs

Compound	IC_{50} (μM)
Curcumin-like	
1	6.06 ±0.36
2	10.5
6	~2.60
16	>40
17	2.66
18	3.00
20	~6.74
23	>40
Dialkylated Curcumin	
3	1.84
33	1.30
36	1.287 ± 0.72
37	2.41
38	1.17
39	3.88
40	1.53
41	2.16
42	3.59
43	1.20

50	1.10
51	2.57
53	2.07
54	1.011 ± 0.41
55	0.708 ± 0.41
56	8.55
57	1.81
58	3.93
Heteroaromatic/aromatic	
4	17.9
5	3.64
29	19.1
30	1.824 ± 1.9
31	>40
34	4.45
35	11.0
47	0.512 ± 0.086
48	7.03
60	4.45
61	5.20
81	5.08
82	5.03
Monocarbonyls	

7	4.62
8	7.67
9	2.90
10	4.10
11	2.70
12	1.249 ± 0.88
13	1.329 ± 1.2
14	9.73
15	16.7
19	>40
21	>40
22	1.11
24	13.8
25	1.682 ± 1.5
28	~18.3
32	1.352 ± 0.69
49	1.56
52	1.87
62	12.9
63	1.67
64	0.488 ± 0.32
66	>40
67	4.32

68	1.14
69	>40
70	6.73
71	>40
79	2.91
83	9.72
Amides	
26	>40
27	12.0
65	>40
72	>40
73	14.6
74	7.27
75	>40
76	7.99
77	13.6
78	>40
80	>40
84	1.42
85	2.49
86	7.51
87	10.1
88	>40

89	>40
90	2.91
91	3.61
92	11.9
93	12.9
94	8.51
95	29.6
96	12.9
97	>40
99	>40
Coumarins	
104	>40
105	>40
105	>40
110	>40
Imides	
98	>40
100	32.2
101	>40
102	>40
103	>40
107	>40
108	>40

109	~16.6
Miscellaneous	
44	8.70
45	4.14
46	3.96

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